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Spermatozoon ultrastructure of Thysanotaenia congolensis (Cyclophyllidea, Anoplocephalidae, Inermicapsiferinae): phylogenetic implications

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Corresponding Author:	Jordi Miquel, Ph.D. University of Barcelona Barcelona, Barcelona SPAIN
Corresponding Author Secondary Information:	
Corresponding Author's Institution:	University of Barcelona
Corresponding Author's Secondary Institution:	
First Author:	Jordi Miquel, Ph.D.
First Author Secondary Information:	
Order of Authors:	Jordi Miquel, Ph.D. Zdzislaw Swiderski Carlos Feliu
Order of Authors Secondary Information:	
Author Comments:	Dear Prof. Mehlhorn, We have made all the modifications suggested by referees. Sincerely yours, Jordi Miquel

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We have made all the modifications suggested by referees.

Sincerely yours,

Jordi Miquel

Spermatozoon ultrastructure of *Thysanotaenia congolensis* (Cyclophyllidea, Anoplocephalidae, Inermicapsiferinae): phylogenetic implications

Jordi Miquel[1,2], Zdzisław Świderski[3], Carlos Feliu[1,2]

[1]Departament de Biologia, Sanitat i Medi ambient, Facultat de Farmàcia i Ciències de l'Alimentació, Universitat de Barcelona, Av. Joan XXIII, sn, 08028 Barcelona, Spain.
[2]Institut de Recerca de la Biodiversitat (IRBio), Universitat de Barcelona, Av. Diagonal, 645, 08028 Barcelona, Spain.
[3]W. Stefański Institute of Parasitology, Polish Academy of Sciences, 51/55 Twarda Street, 00-818 Warszawa, Poland.

Corresponding author:
Jordi Miquel
Secció de Parasitologia, Departament de Biologia, Sanitat i Medi ambient, Facultat de Farmàcia i Ciències de l'Alimentació, Universitat de Barcelona, Av. Joan XXIII, sn, 08028 Barcelona, Spain
e-mail: jordimiquel@ub.edu
Telephone: +34 93 402 45 00
Fax: +34 93 402 45 04

Dedication: This paper is dedicated to the memory of Professor Roger Fons (1942-2016), the distinguished French mammalogist and President of the Federation of Catalan Nature Reserves (France).

Abstract: The mature spermatozoon of *Thysanotaenia congolensis*, an intestinal parasite of black rats *Rattus rattus* from Cape Verde, is described by means of transmission electron microscopy. The ultrastructural organization of the sperm cell of *T. congolensis* follows Levron et al.'s type VII of the Eucestoda. It corresponds to a uniflagellate spermatozoon that presents crested bodies, periaxonemal sheath and intracytoplasmic walls, spiralled cortical microtubules and nucleus spiralled around the axoneme. These characteristics are also present in the spermatozoa of other inermicapsiferines and differ from the characters found in species belonging to the remaining subfamilies of anoplocephalids, namely Anoplocephalinae, Linstowiinae and Thysanosomatinae. Several authors consider the family Anoplocephalidae as a polyphyletic group and its relationships with the Davaineidae are a matter of controversy. The phylogenetic implications of spermatological ultrastructural features present in inermicapsiferines and in the remaining anoplocephalids are discussed and the available data on anoplocephalids are compared to similar results in davaineids in order to contribute to a better knowledge of relationships between these cyclophyllidean families.

Keywords: *Thysanotaenia congolensis*, Cestoda, Cyclophyllidea, Anoplocephalidae, Inermicapsiferinae, sperm characters, ultrastructure

Introduction

Tapeworms of the genus *Thysanotaenia* have traditionally been classified within the subfamily Inermicapsiferinae, which has usually been considered as part of the family Anoplocephalidae (Spasskii 1951; Beveridge 1994). However, there is considerable morphological evidence to suggest that the four currently recognized subfamilies of Anoplocephalidae, namely the Anoplocephalinae, Linstowiinae, Inermicapsiferinae and Thysanosomatinae, represent a polyphyletic assemblage (see Beveridge 1994). Species of the Anoplocephalidae present different uterine structures, such as (i) a tubular or reticular uterus in anoplocephalines, (ii) parenchymatous egg capsules in inermicapsiferines, (iii) single parenchymatous eggs in linstowiines, or (iv) with paruterine organ in thysanosomatines. Cestodes belonging to the Inermicapsiferinae are morphologically similar and seem to be closely related to those included in the family Davaineidae, particularly when consider the uterine structure constituted by fibrous parenchymatous capsules embedding several eggs. However, species of Inermicapsiferinae and Davaineidae differ basically in the absence of rostellum and hooks in the scolex in the case of inermicapsiferines. Diverse authors, such as Lopez-Neyra (1954),

Lopez-Neyra (1955), Baer (1956) or Joyeux and Baer (1961) considered these uterine structures rather than rostellar hooks in the systematics of these families and thus, some genera were transferred between Anoplocephalidae and Davaineidae (see Beveridge 1994). In fact, Baer (1956) considered the inermicapsiferine genera *Inermicapsifer* and *Thysanotaenia* included in the subfamily Davaineinae (Linstowiidae) (see Beveridge 1994). However, there was no consensus among taxonomists in later classifications.

Based on comparative morphology, Hoberg et al. (1999) demonstrate the polyphyly of the Anoplocephalidae with two subclades corresponding to the subfamilies Anoplocephalinae + Thysanosomatinae and Linstowiinae + Inermicapsiferinae. The same authors do not support placing the Inermicapsiferinae as an unarmed group within the Davaineidae, as proposed by Spasskii (1951).

In this context, the ultrastructural characters found in the mature spermatozoon of inermicapsiferines would be additional and useful criteria for a better understanding of relationships between these cestode families. The analysis of ultrastructural characters of the spermatozoon has largely been used for its potential in phylogenetical inference in diverse groups of parasitic Platyhelminthes (Cercomeria), particularly in the Monogenea and Eucestoda (Euzet et al. 1981; Świderski 1986; Bâ and Marchand 1995; Justine 1991a, b, 1995, 1998, 2001; Levron et al. 2010). Over the last years, the integration of both morphology (induding ultrastructural characters) and molecular data has led to a better understanding of relationships within the Cestoda (Hoberg et al. 2001; Olson et al. 2001; Waeschenbach et al. 2007).

Numerous studies have been published concerning ultrastructural characteristics of sperm cells for species belonging to the family Anoplocephalidae, but very little information are available in the case of the subfamily Inermicapsiferinae, for which data is available only concerning the genus *Inermicapsifer* (see Bâ and Marchand 1994a). Thus, the aim of the present work is to provide the first results on the spermatozoon ultrastructure of the genus *Thysanotaenia* with the study of *T. congolensis*. Moreover, we compare all the available spermatological data on anoplocephalid and davaineid species in order to establish the pattern of spermatozoa organization for these two families.

Materials and methods

Live specimens of *Thysanotaenia congolensis* were isolated from the intestine of naturally infected black rats *Rattus rattus* captured in December 2009 in São Domingos and Orgãos (Santiago Island, Cape Verde).

After extraction, adult tapeworms were immediately rinsed with a 0.9% NaCl solution. Later, they were fixed in cold (4 ºC) 2.5% glutaraldehyde in a 0.1 M sodium cacodylate buffer at pH 7.4 for a minimum of 2 h, rinsed in 0.1 M sodium cacodylate buffer at pH 7.4, post-fixed in cold (4 ºC) 1% osmium tetroxide with 0.9% potassium ferricyanide [$K_3Fe(CN)_6$] in the same buffer for 1 h, rinsed in Milli-Q water (Millipore Gradient A10), dehydrated in an ethanol series and propylene oxide, embedded in Spurr's resin and polymerized at 60 ºC for 72 h. Ultrathin sections (60-90 nm thick) were obtained with a Reichert-Jung Ultracut E ultramicrotome. Sections were placed on 200-mesh copper grids and double-stained with uranyl acetate and lead citrate according to Reynolds (1963). The grids were examined in a JEOL 1010 TEM operated at 80kV, in the "Centres Científics i Tecnològics" of the University of Barcelona (CCiTUB).

The periodic acid-thiosemicarbazide-silver proteinate (PA-TSC-SP) technique of Thiéry (1967) was applied to determine the cytochemical localisation of glycogen at the ultrastructural level. Ultrathin sections collected on gold grids were also examined in a JEOL 1010 TEM operated at an accelerating voltage of 80 kV.

Results

The observation and interpretation of numerous cross- and longitudinal sections of sperm cells of *Thysanotaenia congolensis* allow us to establish three distinctive regions from the anterior to the posterior spermatozoon extremity according to the presence of different ultrastructural characters (Figs. 1, 2 and 3I-III).

Region I (Figs. 1 and 3I) corresponds to the anterior extremity of the spermatozoon. The sperm cell is anteriorly capped by a slightly electron-dense apical cone (Figs. 1A, B and 3I). The apical cone (about 900 nm long) is observed from the anterior tip of the spermatozoon to the centriole. Region I is characterized by the presence of two helical electron-dense cords of different lengths, the so-called crested bodies that externally surround the sperm cell (Figs. 1 and 3I). In anterior areas of region I there are two crested bodies very close to one another (Figs. 1A, B and 3I). These two crested bodies progressively separate from each other (Figs. 1C and 3I) and in posterior areas of region I only a single crested body persists (Figs. 1B, E and 3I). The maximum thickness of the crested bodies is about 80 nm in anterior areas of region I. The crested bodies' thickness decreases progressively toward the end of region I. The axoneme, of the 9+'1' trepaxonematan pattern (Figs. 1B, C and 3I), is centrally located and it is surrounded by a thin layer of electron lucent cytoplasm and by an electron-dense submembranous layer of cortical microtubules. The latter are spirally arranged in a 45° angle in relation to the

hypothetical longitudinal axis of the spermatozoon. In posterior areas of this region, a periaxonemal sheath surrounding the axoneme appears very close to the layer of cortical microtubules (Figs. 1B, E and 3I).

Region II (Figs. 1E, 2A–F, K and 3II) corresponds to the middle region of the spermatozoon and it is characterized by the presence of a periaxonemal sheath and by the gradual appearance of intracytoplasmic walls (Figs. 1E, 2A–F and 3II). The intracytoplasmic walls consist in transverse structures that connect the periaxonemal sheath with the peripheral layer of spiralled cortical microtubules. In posterior areas of region II, a large amount of granules appear between the rods of intracytoplasmic walls (Figs. 2E, F, K and 3II). The test of Thiéry evidences the glycogenic nature of this granular material (Fig. 2K).

Region III (Figs. 2G–J and 3III) corresponds to the nuclear or posterior extremity of the spermatozoon. The nucleus forms a loose spiral around the axoneme, presenting a horseshoe shape in both anterior and posterior areas of region III (Figs. 2G, I and 3III) and an annular shape in the middle area where the nucleus encircles the axoneme more than once (Figs. 2H and 3III). The granules of glycogen, the periaxonemal sheath, the intracytoplasmic walls and the cortical microtubules gradually disappear along this region towards the posterior spermatozoon tip (Figs. 2G–J and 3III).

Discussion

The spermatozoon of *Thysanotaenia congolensis* is a filiform cell, which lacks mitochondrion as in all the studied eucestodes. In fact, the absence of mitochondrion in the sperm cell was postulated as a synapomorphy for the Eucestoda (Justine 1998, 2001).

Levron et al. (2010) established seven types of sperm cells in the eucestodes considering different ultrastructural characters such as the number of axonemes, the presence or absence of crested bodies, periaxonemal sheath and intracytoplasmic walls and the parallel or spiralled pattern of cortical microtubules and nucleus. From these patterns of spermatozoa, only four are characteristic of cyclophyllideans (IV to VII). In the case of anoplocephalids and davaineids, spermatozoon types V, VI and VII were described (see Table 1). These three spermatozoal types present a single axoneme, crested body or bodies and a spiralled pattern for both nucleus and cortical microtubules. Type V spermatozoon is additionally characterized by the absence of both periaxonemal sheath and transverse intracytoplasmic walls and it has been described in some anoplocephalids and in hymenolepidids, probably being the typical pattern for nematotaeniids. Type VI spermatozoon is characterized by the additional presence of a

periaxonemal sheath and it has been found in several species of Anoplocephalidae and also in the Catenotaeniidae, Dilepididae, Dipylidiidae and Gryporhynchidae. Finally, type VII spermatozoon exhibits the simultaneous presence of a periaxonemal sheath and intracytoplasmic walls. They have been described in diverse anoplocephalids, in davaineids, in metadilepidids, in paruterinids, in taeniids, and also in species of the order Tetrabothriidea.

There are particularly interesting differences in the ultrastructural organization of sperm cells among anoplocephalid subfamilies. As shown in Table 1, all the analysed anoplocephalines (nine species belonging to eight genera) exhibit the type V spermatozoon. The sole linstowiine studied to date, *Mathevotaenia herpestis* (Bâ and Marchand 1994b), presents a type VI spermatozoon (see Table 1). Concerning the thysanosomatines, there is a great variability in sperm characters. Thus, for the three studied species three different patterns of sperm cells are described (V, VI and VII –see Table 1). Finally, the spermatozoa of the inermicapsiferines analysed to date, *Inermicapsifer guineensis*, *I. madagascariensis* and *Thysanotaenia congolensis* (Bâ and Marchand 1994a; present study –see Table 1), follow the spermatozoon pattern VII. Thus, the ultrastructural organization of the sperm cell of *T. congolensis*, as for the *Inermicapsifer* species, corresponds to a uniflagellate spermatozoon that presents crested bodies, periaxonemal sheath and intracytoplasmic walls, spiralled cortical microtubules and nucleus spiralled around the axoneme. Evidently, the great variability in the spermatozoon organization of different anoplocephalids corroborates the polyphyletic status of this cyclophyllidean family, as postulated by several authors considering morphological characters (Beveridge 1994; Hoberg et al. 1999).

Taking into account all the above mentioned ultrastructural characteristics of sperm cells and considering the systematic status of anoplocephalids and davaineids, it is crucial to compare the present results with the ultrastructure of spermatozoa of the studied species of the family Davaineidae. These are *Cotugnia polyacantha, Paroniella reynoldsae, Raillietina baeri, R. micracantha* and *R. tunetensis* (for references see Table 1). All five species present a type VII spermatozoon as occurs in the three inermicapsiferines studied to date.

Differences between anoplocephalid subfamilies are also evident when considering data on spermiogenesis. Spermiogenesis in cestodes is characterized by diverse aspects, particularly (i) the formation of two free flagella, only one flagellum or an axoneme from the centrioles present in the zone of differentiation, (ii) the presence or absence of flagellar rotation between the flagellum or flagella and the cytoplasmic process and (iii) the presence or absence of proximodistal fusion between the flagellum or flagella and the cytoplasmic process (see Świderski 1968; Justine 1991, 1998; Bâ and Marchand 1995). Thus, according to these three

main aspects and other additional characteristics, Bâ and Marchand (1995) established four types of spermiogenesis in cestodes (1 to 4). Except for mesocestoidids (see Miquel et al. 1999, 2007), all the cyclophyllideans follow type 3 or type 4 spermiogenesis. It seems that species with a pattern V spermatozoon follow a type 4 spermiogenesis, which is characterized by the formation of a single axoneme that elongates directly into the spermatid body and, consequently, no flagellar rotation nor proximodistal fusion occur. This is the case of six anoplocephalines, namely *Anoplocephaloides dentata*, *Aporina delafondi*, *Gallegoides arfaai*, *Moniezia expansa*, *Mosgovoyia ctenoides* and *Sudarikovina taterae* (for references see Table 1) and the thysanosomatine *Thysaniezia ovilla* (Bâ et al. 1991). The linstowiine *Mathevotaenia herpestis* (Bâ and Marchand 1994b) presents a type 3 spermiogenesis. Type 3 spermiogenesis is characterized by the formation of a free flagellum that grows externally to the cytoplasmic process and by the proximodistal fusion of these two processes. Unfortunately, there are no data concerning spermiogenesis for the inermicapsiferines (see Table 1). Considering the Davaineidae, two of the five species studied to date, namely *R. micracantha* (Miquel et al. 2005) and *R. tunetensis* (Bâ and Marchand 1994c) exhibit the type 3 spermiogenesis (see Table 1). Moreover, other cyclophyllideans presenting sperm cells of type VII also follow the type 3 spermiogenesis. This is the case of the only metadilepidid studied to date, namely *Skrjabinoporus merops* (Yoneva et al. 2006), the paruterinids *Anonchotaenia globata*, *Notopentorchis* sp. and *Triaenorhina rectangula* (Yoneva et al. 2009, 2010, 2012), and the taeniids *Taenia parva* and *T. taeniaeformis* (Ndiaye et al. 2003; Miquel et al. 2009). All these results seem to indicate that the formation process of type VII spermatozoa corresponds to the pattern 3 of spermiogenesis, and this pattern is probably present in inermicapsiferines.

Recently, a complete ultrastructural study of the parenchymatic capsules, eggs and cellular organization of hexacanths of *T. congolensis* has been performed (Świderski et al. 2015, 2016). Important differences in eggs and hexacanths of the inermicapsiferines *T. congolensis* and *I. madagascariensis* (Świderski and Tkach 2002; Świderski et al. 2015, 2016) in comparison to data on linstowiines and particularly on anoplocephalines (see Świderski et al. 2016) clearly support the phylogenetic distinction between *T. congolensis* and cestodes of the subfamily Anoplocephalinae. With respect to the family Davaineidae, unfortunately, there are no ultrastructural studies on eggs and hexacanths. These would be of great interest to corroborate ultrastructural similitudes of uterine characters in inermicapsiferines and davaineids as already demonstrated in sperm characters.

Conclusion

Our new ultrastructural data on sperm organization and its comparison with previous studies on other anoplocephalids and also davaineids provide new evidences that *T. congolensis* and other inermicapsiferines are closely related to the family Davaineidae. Sperm cells of *T. congolensis* and *Inermicapsifer* spp. present typical characters of davaineids, a type VII sperm cell, mainly characterized by intracytoplasmic walls. The implication is that *Thysanotaenia*, and perhaps all inermicapsiferines, are actually davaineid cestodes that have secondarily lost their rostellar armature (see Khalil et al. 1994). The present ultrastructural data, in fact, support the phylogenetic distinction between Inermicapsiferinae and Anoplocephalinae and Linstowiinae (Hoberg et al. 1999). Moreover, new ultrastructural data concerning spermatozoa would be needed for other thysanosomatines to evaluate and corroborate or not the great diversity in the sperm patterns observed in this subfamily. Taking into account the evident relation between different patterns of spermiogenesis and spermatozoon types, future studies on spermiogenesis in inermicapsiferines are also necessary. Overall, our ultrastructural results provide a significant contribution to understanding the phylogenetic relationships among these cyclophyllidean families.

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Legends to figures

Fig. 1 Anterior region (I) of the mature spermatozoon of *Thysanotaenia congolensis*. **A** Longitudinal section of region I showing the anterior spermatozoon extremity. **B–D** Cross-and longitudinal sections showing the aspect of crested bodies at different levels (* apical cone level). **E** Longitudinal section showing the transitional area between regions I and II. *AC* apical cone, *ASE* anterior spermatozoon extremity, *C* centriole, *CB* crested bodies, *CM* cortical microtubules, *PS* periaxonemal sheath.

Fig. 2 Middle and posterior regions (II and III) of the mature spermatozoon of *Thysanotaenia congolensis*. **A** Longitudinal section of region II showing the gradual appearance of intracytoplasmic walls. **B and C** Cross-sections of anterior and middle areas of region II. **D and E** Longitudinal sections of middle and posterior areas of region II. **F** Cross-section of posterior area of region II. **G–J** Consecutive cross-sections of nuclear region towards the posterior spermatozoon extremity. **K** Cross-sections showing the positive labelling of glycogen by means of the test of Thiéry. *CM* cortical microtubules, *D* doublets, *G* glycogen, *IW* intracytoplasmic walls, *N* nucleus, *PS* periaxonemal sheath.

Fig. 3 Schematic reconstruction of the mature spermatozoon of *Thysanotaenia congolensis*. *AC* apical cone, *ASE* anterior spermatozoon extremity, *Ax* axoneme, *C* centriole, *CB* crested bodies, *CM* cortical microtubules, *D* doublets, *G* glycogen, *IW* intracytoplasmic walls, *N* nucleus, *PAE* posterior axonemal extremity, *PM* plasma membrane, *PS* periaxonemal sheath, *PSE* posterior spermatozoon extremity.

Table 1. Ultrastructural characters of spermiogenesis and the spermatozoon in the Anoplocephalidae and Davaineidae cestodes.

Family, subfamily, species	Spermiogenesis						Spermatozoon								References
									CB						
	Type[a]	VSR	IB	FR	PF	CA	Type[b]	AC (nm)	n	Thickness (nm)	CM	PS	G	IW	
ANOPLOCEPHALIDAE															
Anoplocephalinae															
Anoplocephaloides dentata	4	+	-	-	-	-	V	1400	2	140	30	-	+	-	Miquel and Marchand (1998a)
Aporina delafondi	4	-	-	-	-	-	V	300	5	15-40	15	-	+	-	Bâ and Marchand (1994d)
Gallegoides arfaai	4	+	-	-	-	+	V	1000	2	140	35	-	+	-	Miquel et al. (2004, 2005)
Moniezia benedeni							V	1000	2	30-40	spi	-	+	-	Bâ and Marchand (1992a)
M. expansa	4	+	-	-	-	-	V	1000	2	30-60	40	-	+	-	Bâ and Marchand (1992a)
Monoecocestus americanus							V	?	2	spi	30-35	-	+	-	MacKinnon and Burt (1984)
Mosgovoyia ctenoides	4	+	-	-	-	+	V	+	2	70-90	30	-	+	-	Eira et al. (2006)
Paranoplocephala omphalodes							V	900	2	180	25-35	-	+	-	Miquel and Marchand (1998b)
Sudarikovina taterae	4	-	-	-	-	+[c]	V	500	7	50-100	spi	-	+	-	Bâ et al. (2000)
Inermicapsiferinae															
Inermicapsifer guineensis							VII	+	2	40	30-35	+	-	+	Bâ and Marchand (1994a)
I. madagascariensis							VII	+	2	40	30-35	+	-	+	Bâ and Marchand (1994a)
Thysanotaenia congolensis							VII	900	2	80	45	+	-	+	Present study
Linstowiinae															
Mathevotaenia herpestis	3	-	-	-	+	+[c]	VI	+	1	70	40	+	-	-	Bâ and Marchand (1994b)
Thysanosomatinae															
Avitellina centripunctata							VII	700	1	150-200	35	+	-	+	Bâ and Marchand (1994e)
Stilesia globipunctata							VI	1250	1	150	50	+	-	-	Bâ and Marchand (1992b)
Thysaniezia ovilla	4	-	-	-	-	+	V	600	2	80	40-50	-	+	-	Bâ et al. (1991)
DAVAINEIDAE															
Cotugnia polyacantha							VII	+	2	50-100	spi	+	-	+	Bâ and Marchand (1994f)
Paroniella reynoldsae							VII	2200	2	100-150	45	+	-	+	Bâ et al. (2005a)
Raillietina baeri							VII	2500	2	100-125	50	+	-	+	Bâ et al. (2005b)
R. micracantha	3	-	-	-	+	-	VII	1000	2	100	50	+	-	+	Miquel et al. (2010)
R. tunetensis	3	-	-	-	+	-	VII	+	2	100-200	60	+	-	+	Bâ and Marchand (1994c)

AC length of apical cone, *CA* centriolar adjunct, *CB* crested bodies, *CM* angle of cortical microtubules, *FR* flagellar rotation, *G* electron-dense granules, *IB* intercentriolar body, *IW* intracytoplasmic walls, *n* number, *PF* proximodistal fusion, *PS* periaxonemal sheath, *spi* spiralled, *VSR* vestigial striated rootlets, *+/-* presence/absence of considered character, *?* unknown character.

[a]Type of spermiogenesis according to Bâ & Marchand (1995).

[b]Type of spermatozoon according to Levron et al. (2010).

[c]Particular types of centriolar adjuncts.

Figure 1

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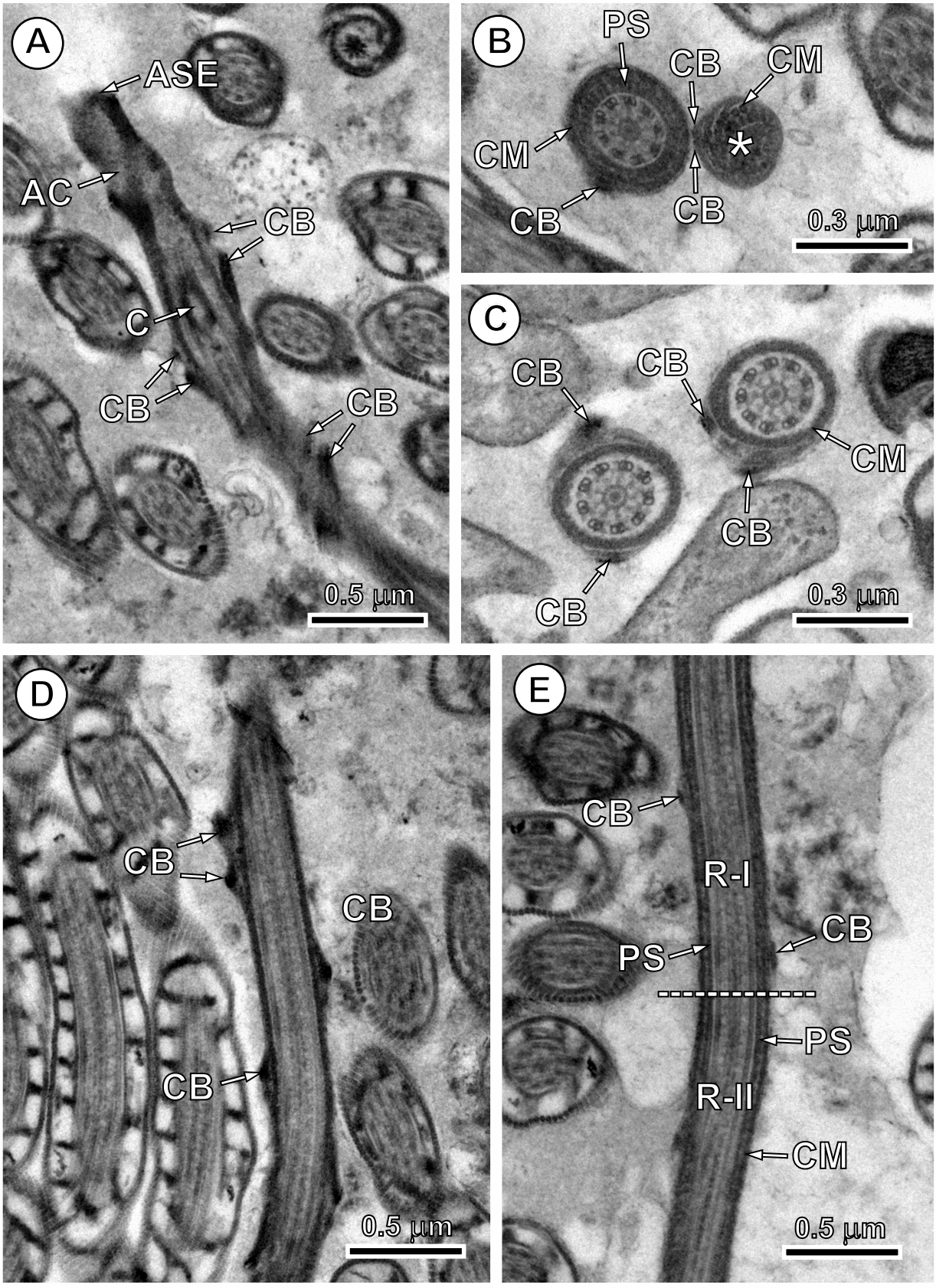

Figure 2

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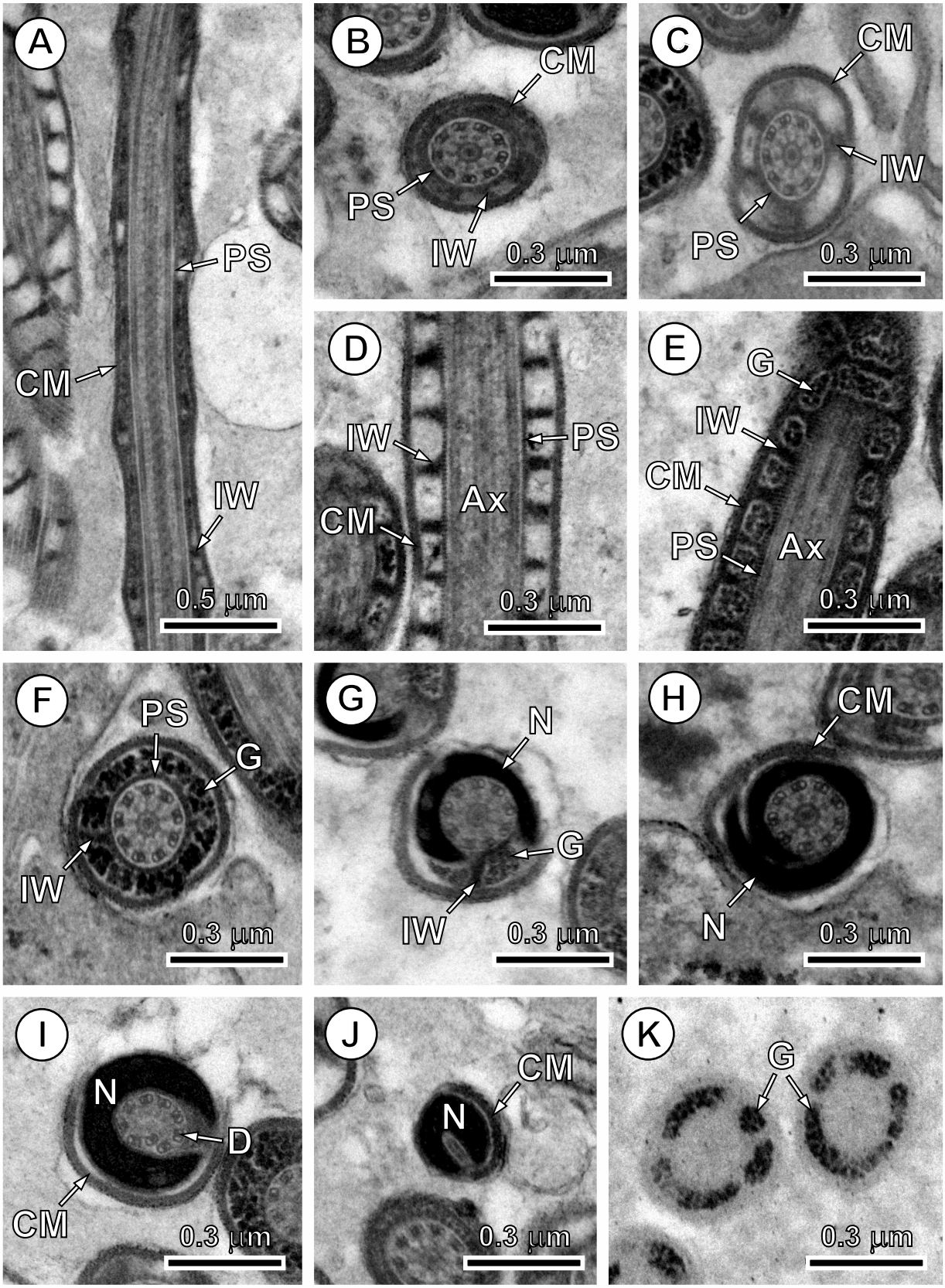

Figure 3

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